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METHODOLOGICAL ASPECTS OF BACTERIAL PROTEIN DETERMINATION IN SWINE DIGESTIVE SAMPLES

BY

MICHAEL EDWARD RUSSELL DUGAN ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

IN

ANIMAL NUTRITION

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA

FALL 1992

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled METHODOLOGICAL ASPECTS OF BACTERIAL PROTEIN DETERMINATIONS IN SWINE DIGESTIVE SAMPLES submitted by MICHAEL EDWARD RUSSELL DUGAN in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ANIMAL NUTRITION.

W.C. Sauer

J. Sim

Signed f. J.R. Thompson.

J.R. Thompson

L. Ozimek

R R Marquardt

R.R. Marquardt

Oct. 6, 1992

DEDICATION

TO MY PARENTS

JOHN AND ANNA DUGAN

FOR YOUR YEARS OF SACRIFICE AND SUPPORT
AND
THE ROAD HOME WHEN I NEEDED IT

ABSTRACT

A series of experiments were conducted to determine the bacterial contribution to protein in digestive samples collected from the pig. In the first experiment, pigs were fed either a conventional barley/wheat/soybean meal or a purified casein/corn starch grower diet. Sulfur-35 was used to label the hydrolysis and oxidation stable organic fraction of the gastrointestinal bacteria to determine the portion of crude protein (CP) in ileal digesta and feces contributed by bacteria. The majority of the estimates of the bacterial contribution to CP in both ileal digesta and feces yielded values that exceeded 100%. Therefore, a second study was conducted, in which a refined ^{35}S marker technique was used in conjunction with a second bacterial marker, diaminopimelic acid (DAPA).

Prior to the commencement of the dual marker study, two technical problems had to be overcome. Analyses in the original ^{35}S study were slowed due to the lack of appropriate drying equipment for use with test tubes. A vortex vacuum evaporator for test tubes was subsequently constructed to fulfill anticipated needs. A micro-method was then developed for DL-DAPA analysis involving cellulose clean-up and high performance liquid chromatography (HPLC).

The dual marker study was conducted to determine the bacterial contribution to amino acids in ileal digesta in pigs fed a conventional barley/wheat/soybean grower diet.

The bacterial contribution to amino acids in ileal digesta was calculated to be 29.4% when using the DAPA marker and 191% when using the ^{35}S marker. Based on the DAPA marker technique, bacteria were found to significantly ($P < .05$) affect the percentages of 5 of the 16 amino acids measured in ileal digesta. In addition, when bacterial amino acid levels in ileal digesta changed within pig over time, the undigested endogenous and dietary residual amino acids levels changed in the same direction.

Difficulties encountered with DL-DAPA measurement in the dual marker study stimulated the development of a second micro-method for DAPA analysis. The newer method utilizes the ion-pairing agent hexadecyl-trimethyl-ammonium bromide to enable the simultaneous measurement of both DAPA peaks (DD,LL-DAPA and DL-DAPA). With this method, either DAPA enantiomer can be measured to below 82 ng per mg of digesta (CV < 4% for either DAPA enantiomer analyzed in physiolgical samples).

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Table of Contents

Chapter Page

I. INTRODUCTION 1

A. BIBLIOGRAPHY 11

II. DETERMINATION OF THE BACTERIAL CONTRIBUTION TO CRUDE PROTEIN IN PIG ILEAL DIGESTA AND FECES USING A ^{35}S MARKER TECHNIQUE 16

A. INTRODUCTION 17

B. EXPERIMENTAL PROCEDURE 17

C. RESULTS AND DISCUSSION 21

D. BIBLIOGRAPHY 30

III. CELLULOSE CLEANUP AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF DL-DIAMINOPIMELIC ACID IN HYDROLYSATES OF PHYSIOLOGICAL SAMPLES 32

A. INTRODUCTION 32

B. EXPERIMENTAL PROCEDURE 33

C. RESULTS AND DISCUSSION 37

D. BIBLIOGRAPHY 42

IV. A VORTEX VACUUM EVAPORATOR FOR SAMPLES IN TEST TUBES 44

A. INTRODUCTION 44

B. EXPERIMENTAL PROCEDURE 45

C. RESULTS AND DISCUSSION 47

V. DETERMINATION OF THE BACTERIAL CONTRIBUTION TO AMINO ACIDS IN PIG ILEAL DIGESTA USING A ^{35}S AND A DAPA MARKER TECHNIQUE 51
A. INTRODUCTION 51
B. MATERIALS AND METHODS 53
C. RESULTS AND DISCUSSION 61
D. BIBLIOGRAPHY 73

VI. ION-PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF DIAMINOPIMELIC ACID IN HYDROLYSATES OF PHYSIOLOGICAL SAMPLES 77
A. INTRODUCTION 77
B. MATERIALS AND METHODS 79
C. RESULTS AND DISCUSSION 83
D. BIBLIOGRAPHY 89

VII. GENERAL DISCUSSION AND CONCLUSIONS 91
A. BIBLIOGRAPHY 96

List of Tables

Table Page

II.1 Composition of diets (%) 27

II.2 Organic ^{35}S activity in bacterial, ileal digesta and fecal CP ($Bq \cdot mg^{-1}$ CP) 28

II.3 Percent bacterial CP in total CP in ileal digesta and feces 29

III.1 HPLC gradient conditions 39

V.1 HPLC solvent gradient for DAPA analysis 67

V.2 HPLC solvent gradient for analysis of sulfur containing amino acids 68

V.3 Percent of total ileal amino acids arising from bacteria 69

V.4 Partitioning of the total dietary amino acids (%) 70

V.5 Amino acid composition of ileal bacteria (B) and ileally undigested endogenous and dietary residuals (R) expressed as a percentage of total ileal digesta amino acids 71

V.6 Regression coefficients (β_1) for individual amino acids (apparent ileal amino acid digestibility (%)(Y) regressed versus the ileal bacterial content of dietary amino acid (%)(X)) 72

VI.1 HPLC gradient conditions 86

List of Figures

Figure Page

III.1 Chromatograms of the simulated 15% CP sample with a 13.5 nmol DL-DAPA addition per mL (before cellulose cleanup): (a) without cellulose cleanup (b) with cellulose cleanup. Peaks: 1 = DL-β-amino-n-butyric acid, 2 = LL and DD-DAPA (included in the DL-α,ϵ-diaminopimelic acid standard), 3 = DL-DAPA, 4 = methionine, 5 = valine, 6 = phenylalanine 40

IV.1. A heat assisted vortex vacuum evaporator for small liquid samples 49

VI.1. Chromatogram of the rumen bacteria hydrolysate. Peaks: 1 = DL-α-amino-caprylic acid; 2 = DAPA I; 3 = DAPA II 87

I. INTRODUCTION

Swine require balanced diets that provide adequate levels of all nutrients, including amino acids. In order to provide adequate, but not excessive levels of amino acids to swine, the digestible rather than the total amino acid supply should be considered.

As reviewed by Sauer and Ozimek (1986), changes in methods to determine the amino acid supply have closely paralleled the elucidation of factors causing aberrations in the supply. Measuring the supply of amino acids to the pig by simply determining the content of amino acids in the diet was replaced by use of the fecal analysis method, developed by Kuiken and Lyman (1948). The fecal analysis method measures the difference between the quantity of amino acids consumed and excreted in feces. However, studies thereafter showed that the ileal rather than fecal analysis method should be used to determine the supply. The ileal analysis method measures the differences between the quantity of amino acids consumed and recovered in digesta collected from the distal ileum. Zebrowska (1973) originally demonstrated that amino acids in the large intestine are degraded prior to their absorption to yield little or no benefit to the nitrogen economy of the pig. In addition, depending on the supply of fermentable carbohydrate, the bacteria in the large intestine may considerably modify both the profile and quantity of amino acids excreted in feces (Mason, 1984).

The presence and activity of bacteria proximal to the large intestine have been dismissed in the past as having little effect on the amino acid supply to the pig. The validity of the ileal analysis method is based on the assumption that only negligible bacterial transformations of amino acids occur in digesta proximal to the large intestine (Low and Zebrowska, 1989). It was assumed that hydrochloric acid secreted into the lumen of the stomach was able to effectively sterilize ingesta and thus eliminate any bacterial effect (Hungate, 1984).

Volatile fatty acids (VFA's) are end products of bacterial metabolism of carbohydrate and protein; their concentrations in digesta are thus indicators of bacterial activity. Concentrations of VFA's were found to be lower in ileal than cecal digesta, approximately 8-10 fold (Clemens et al., 1975; Chesson et al., 1985). However, the lower VFA concentrations may have been due to the high absorptive capacity per unit length of the small intestine. Graham et al. (1985, 1986) concluded that between 45 and 60% of the digestion of non-starch polysaccharides occurs proximal to the large intestine when pigs were fed cereal based diets. High digestibilities were mainly due to elevated mixed-linked β-glucan digestion. When increased insoluble non-starch polysaccarides were incorporated into the diet (e.g.

from wheat bran), digestion of non-starch polysaccarides proximal to the large intestine decreased to approximately 20 to 40% (Graham et al., 1985; Vervaeke et al., 1991). The total number of bacteria were found to increase sharply towards the end of the small intestine when pigs were fed a purified casein/corn starch based diet (Wilbur et al., 1960). Counts of bacteria in ileal digesta were, however, approximately 10 fold less than in cecal digesta. In contrast, Chesson et al. (1985) found approximately equal numbers of bacteria in ileal and cecal digesta when pigs were fed a diet containing bran as the fibre source. As indicated by Liu et al. (1985), the bacterial population throughout the digestive tract of pigs was composed of a mixture of gram positive (mainly lactobacilli and streptococcal strains) and gram negative (mainly enterobacterial strains) bacterial species when pigs were fed a cereal based diet.

Bacterial activities indicating a more direct effect on the supply of amino acids have been elucidated through the inclusion of sub-therapeutic levels of antibiotics in the diet. Just et al. (1985) found a trend towards increased ileal N (+3%) and lysine (+2%) digestibilities when feeding pigs a barley/wheat/soybean meal diet supplemented with virginiamycin. Dierick et al. (1986) also reported a trend towards increased ileal N (+2.1%) and lysine (+1.4%) digestibilities in studies conducted with pigs fed a skim milk powder/corn starch based diet supplemented with virginiamycin. In addition, Dierick et al. (1986) demonstrated that virginiamycin increased the absorption rate (9%) of free amino acids perfused through an isolated loop of distal ileum. In the same study, Dierick et al. (1986) reported decreased concentrations of primary amines and ammonia in ileal digesta of pigs fed a corn/manioc based diet supplemented with virginiamycin.

Bacterial activities along the digestive tract, as indicated by adenosine triphosphate (ATP) concentration, were shown to be greatest (18 ug ATP per g) in digesta collected from the distal ileum when a wheat flour based diet (4% crude fibre) was fed (Jensen, 1988). When diets were fed containing elevated fibre levels, however, the highest ATP concentrations (35-45 ug ATP per g) were found in cecal digesta (Knudsen and Hanson, 1991; Jensen, 1988). Concentrations of ATP in digestive contents from all sampling sites were found to decrease upon supplementation of the diets with virginiamycin (Jensen, 1988).

The literature reviewed thus far indicates that bacteria are capable of affecting the supply of amino acids absorbed from the lumen of the small intestine. Decarboxylation, deamination and further bacterial metabolism can reduce the quality of the amino acid supply to the pig. In addition, as shown by increased bacterial numbers and thus increased bacterial amino acids in ileal digesta, bacteria can directly reduce the supply of amino acids available to the pig. Bacterial counts, however, only indicate the presence of bacteria and not their actual composition. A step towards understanding the full impact that bacteria have on the amount of amino acids actually absorbed from the small intestine is, therefore, to quantify the bacterial contribution to amino acids in ileal digesta.

The separation of all the bacteria from a particular sample of digesta is impossible. A direct measurement of the bacterial amino acid content in digesta can, therefore, not be made. The collection of a sub-sample of purified bacteria, however, is possible by centrifugation of the digesta at low speed to remove feed particles (and some bacteria) and then at high speed to collect a purified (assumed representative) bacterial pellet. An indirect measure of the bacterial contribution to amino acids in the digesta is then possible through the use of a bacterial marker, i.e. a compound that only the bacteria possess. The bacterial amino acid contribution is calculated by dividing the marker to amino acid ratio in the digesta by the marker to amino acid ratio in the purified bacteria.

A bacterial marker that would provide an absolute determination of the bacterial amino acids in ileal digesta, unfortunately, does not exist. The seemingly simple qualification of a bacterial marker, i.e. that it is only found only in bacteria, disqualifies all known bacterial markers the moment any marker or amino acids are released into digesta upon bacterial lysis. The fate of the released bacterial marker and amino acids may differ leading to an incorrect estimation of the bacterial amino acid levels in the digesta (e.g., if the bacterial protein is digested and absorbed by the pig and most of the marker remains in the digesta, then an overestimation of the bacterial amino acid levels in ileal digesta would result). In the absence of an ideal marker, the only way to estimate the bacterial amino acid levels in ileal digesta is to use a less than perfect marker. Even though such estimations may not provide an absolute determination of the bacterial amino acids in ileal digesta, an estimation of this nature is certainly better than no estimation at all.

The validity of bacterial amino acid estimations also rests on the assumption that a representative purified bacterial pellet is collected from ileal digesta. If a non-representative bacterial pellet is obtained, an incorrect bacterial amino acid level in ileal digesta may result due to differing marker to amino acid ratios within the sub-populations of bacteria in ileal digesta (e.g., fluid associated bacteria may have a different marker to amino acid ratio than feed particle associated bacteria). Every effort should, therefore, be made to increase the chance of collecting a representative bacterial pellet (e.g., the use of a mild detergent to remove bacteria from feed particles, the use of a physiological diluent instead of distilled water for washing the bacterial pellet, etc.).

The pioneering studies to determine the bacterial contributions to crude protein (CP) and amino acids in digestive samples were conducted using ruminant animals. Broderick and Merchen (1992) recently reviewed the positive and negative aspects of the various bacterial markers currently used in studies with ruminant animals. The markers reviewed included the internal markers diaminopimelic acid (DAPA), D-alanine and the nucleic acids (RNA and/or DNA); and the external (isotopic) markers ^{35}S, ^{32}P, and ^{15}N. Fewer bacterial markers can be used in studies with pig ileal digesta due to the increased potential for confounding sources of bacterial markers in dietary and/or endogenous materials (dietary and endogenous nucleic acids and endogenously synthesized ^{15}N labelled amino acids and ^{32}P labelled phospholipids). Of the remaining three markers, DAPA and ^{35}S have been used most extensively in ruminant studies, while D-alanine has received little attention (DAPA pre-dates the use of D-alanine as a marker and both markers were proposed to have the same imperfections, therefore, the use of D-alanine as a bacterial marker has not flourished).

Focusing on the remaining possible markers for use in studies with pigs, DAPA is an amino acid found almost exclusively in bacterial cell walls. Synge (1953) was the first to propose the use of DAPA for a bacterial marker due to its absence in feeds but presence in bacteria in the digestive tract. Sulfur-35 is a radioactive isotope of sulfur; it can be used as a bacterial marker when incorporated into bacterial (oxidation and hydrolysis stable) organic matter from an inorganic source (e.g. SO_4^{2-}). The total, or a fraction of the total (e.g., ^{35}S-methionine sulfone), oxidation and hydrolysis stable ^{35}S labelled organic matter can be used as a bacterial marker due to the lack of mammalian enzymes necessary for SO_4^{2-} reduction and subsequent sulfide incorporation into methionine and cysteine (the two principle S-containing organic molecules in bacteria). Several variations on the ^{35}S marker technique have been used, of which most have been derived from the original study of Beever et al. (1974).

Of the crude and amino acid protein in swine ileal digesta, 20-35% were estimated to be of bacterial origin when pigs were fed cereal-based diets. Dierick et al. (1983) and Poppe et al. (1983) estimated CP while Drochner (1984) estimated total amino acids. These estimates were calculated using diaminopimelic acid (DAPA) as a bacterial marker. Drochner (1984) reported a large variation between pigs for the bacterial contribution to amino acids in ileal digesta; Dierick et al. (1983) and Poppe et al. (1983) failed to report any descriptive statistics. Between animal variations of bacterial CP measurements in ruminant studies were also found to be large when using DAPA as a marker.

This variation, however, was circumvented through the use of a ^{35}S bacterial marker (Ling and Buttery, 1978).

The fundamental objectives of the present studies were to develop, refine and implement methods to determine the bacterial amino acid levels in pig digestive samples (principally in digesta collected from the distal ileum). An initial animal experiment was to be conducted using ^{35}S to estimate the bacterial contributions to CP in pig ileal digesta and feces and determine if the large between animal variation reported by Drochner (1984), when using DAPA as a marker, could be avoided. A subsequent animal experiment was then to be conducted using a combined marker method (^{35}S and DAPA) to determine the bacterial contribution to amino acids in ileal digesta to enable a direct comparison of both marker methods. Prior to the second animal experiment, a micro-method for the analysis of DL-DAPA using high performance liquid chromatography (HPLC) had to be developed (small quantities of purified bacteria collected during the first animal experiment actually limited analyses even without DAPA measurement). A rapid evaporator for use with test tubes also then had to be developed to speed up analyses in the second animal experiment and enable scaling down of analytical procedures. Subsequent to the second animal experiment, a second simpler HPLC method for the micro-analysis of DAPA was investigated to provide more routine analyses of both DAPA enantiomer peaks (DL-DAPA and

DD,LL-DAPA) for use in future animal experiments.

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II. DETERMINATION OF THE BACTERIAL CONTRIBUTION TO CRUDE PROTEIN IN PIG ILEAL DIGESTA AND FECES USING A ^{35}S MARKER TECHNIQUE.

A. INTRODUCTION

Presently, protein sources for swine are often rated on the basis of their supply of digestible amino acids, as measured with the ileal analysis method. The ileal analysis method is preferred to the fecal method because of the modifying action microflora have on amino acids in the large intestine. Studies on the effect of the microflora in the stomach and small intestine of the monogastric animal on the utilization of amino acids have received little attention. Several studies, however, have shown beneficial effects on nutrient utilization when sub-therapeutic levels of antibiotics were included in diets for swine (e.g., Dierick et al., 1986; Yen et al., 1987). Improved pig performance, upon supplementaion of diets with antibiotics, could be linked to reduced bacterial growth and thus their reduced assimilation of dietary amino acids. It is, therefore, of importance to be able to quantify the bacterial contribution to crude protein (CP) in digesta (collected from the distal ileum) to be able to determine their affect on the supply of CP to the pig.

When using the diaminopimelic acid (DAPA) marker technique, 20-35% of the CP and amino acids contained in ileal digesta were estimated to be contributed by bacteria in studies with pigs fed cereal based diets (Dierick et al.,1983; Poppe et al., 1983; Drochner, 1984). Drochner (1984), the only author to provide descriptive statistics, reported large between pig variation in the bacterial contribution to amino acids in ileal digesta. In ruminant studies, estimates of bacterial CP in rumen digesta, using ^{35}S marked bacteria, were less variable and thus seemingly more accurate, than results obtained when DAPA was used for a marker (Ling and Buttery, 1978). The objective of this study was to determine if the method of Mathers and Miller (1980), which uses organically bound ^{35}S as a marker for estimating bacterial CP as a percentage of total CP in rumen digesta, could be applied to make similar measurements in swine ileal digesta and feces.

B. EXPERIMENTAL PROCEDURE

Four barrows (Lacombe x Yorkshire), with an average initial body weight of approximately 45 kg, were surgically fitted with a simple T-cannula in both the stomach and distal ileum. The design of the cannula inserted into the ileum was initially described by Sauer et al. (1983) and later modified by de Lange et al. (1989). The design of the cannula inserted into the stomach was similar the re-entrant arm of the pancreatic cannula described by Hee et al. (1985). The barrows were fasted 24 h before surgery; one hour prior to surgery the animals were sedated with a 1 mL intramuscular injection of Atravet (Ayerst Laboratories, Montreal, Que., Can.). The surgical techniques outlined by Sauer et al. (1983) were modified for the insertion of the cannulas into the lumen of the stomach and ileum. Surgical incision and cannula exteriorizations were performed on the left side of the animals to permit easier access to both the stomach and distal ileum. After surgery, the animals were housed individually in stainless steel metabolic crates in a barn maintained at 20 ^{0}C. The pigs were permitted a 10 day recovery period during which time an 18% CP starter diet (Sauer et al., 1983) was fed twice daily at 0800 and 2000 h. The dietary allowance was gradually increased from 50 g per feeding the day after surgery to ad libitum on day five. Post-surgical pain relief was provided by the addition of Torbugesic (Ayerst Laboratories, Montreal, Que., Can.) to the feed (1.2 mg per kg body weight).

The experiment was conducted using a simple 2 x 2 cross over design comprised of two 14 d experimental periods. The experimental diets included a casein/corn starch purified grower diet and a barley/wheat/soybean meal conventional grower diet (Table II.1). The purified diet included 10% dextrose to possibly improve its palatability. Both diets contained 16.5% CP (as fed) and were offered in 1 kg quantities at 0800 and 2000 h. Water was supplied ad libitum from a low pressure drinking nozzle. From days 9 to 14 of each experimental period, 560 mL of an aqueous solution containing 17.8 MBq of $Na_2{}^{35}SO_4$ (ICN Biomedicals Inc., Irvine, CA, U.S.A.) and 500 mg of $Na_2{}^{32}SO_4$ was continuously infused into the stomach of each pig each day. Fresh feces were collected and frozen immediately at -20 °C on day 13 and the morning of day 14. During day 14 ileal digesta were collected for 24 h according to the procedures described by de Lange et al. (1989). Ileal digesta were collected onto ice in flexible polyethylene tubing (1.5 m length, 4 cm width) with two hourly outflows immediately frozen at -20 °C. After the experiment the animals were euthanized and the cannulation sites inspected for abnormalities.

Bacterial Separations. Frozen ileal digesta were pooled, as were frozen feces, for each pig in each period. Pooling was accomplished by pulverizing the samples together (into fine particles) with a 1 kg aluminium cylinder. Representative bacteria were collected from digesta and feces according to a modification of the method described by Ahrens (1984). Two seperations were completed per sample. For each seperation, either 100 g of digesta or 50 g of feces were combined with 800 mL of .9% NaCl saline, 8 mL 88% formic acid, and 2 mL Triton X-100 in a 1 l centrifuge bottle. The suspensions were mixed at 150 cycles per min for 1 h on a Lab-Line™ Orbit Enviro-Shaker (Lab-Line Instruments Inc., Melrose Park, Ill., U.S.A.) and were centrifuged at 4 °C in a Beckman J-6B/P Centrifuge (Beckman, Palo Alto, CA, U.S.A) for 15 min at 200 *g* to remove feed particles. The supernatants were filtered through several layers of glass wool and centrifuged again for 15 min at 200 *g*. The resultant supernatants were filtered again and centrifuged at 4 °C in a Sorvall Super Speed Refrigerated Centrifuge (Sorvall Inc., Norwalk, CO, U.S.A.) in 250 mL centrifuge bottles at 9100 *g* for 20 min to pellet the bacteria (P.H. Robinson, personal communication). The pellets were washed twice in 800 mL 0.9% saline, with each washing followed by centrifugation at 9100 *g* for 20 min. After the last centrifugation, the pellets were collected for subsequent analyses.

Chemical Analyses. Pooled ileal digesta, feces and bacteria were freeze-dried and ground through a 1 mm mesh screen prior to analysis. Bacteria (200 mg), digesta (350 mg in duplicate) or feces (350 mg in duplicate) were placed in 250 mL round bottom flasks, and prepared for scintillation counting using the method described by Mathers and Miller (1980). Counting was completed using Ecolite™ Scintillation Counting Fluid and a Searl Mark III 6880 Liquid Scintillation System set for low background optimized ^{35}S counting. Samples were counted for enough time to accumulate 10,000 total counts (i.e., 95% confidence level that counting error would be less than ± 2%). Sulfur-35 activity in the samples was then obtained after background and counting efficiency corrections were made. Kjeldahl digestion (AOAC, 1984) and free ammonia determinations (Fawcett and Scott, 1960) were used to estimate the CP content of bacteria, digesta and feces.

Bacterial CP Calculations. In order to calculate the bacterial contribution to total CP in ileal digesta (or feces) two assumptions were made. First, that representative bacteria were collected during differential centrifugation, and second, only bacteria were able to incorporate inorganic sulfur into their organic fraction which was both stable to peroxidation and hydrolysis. The bacterial contribution to CP in digesta (or feces) was then calculated by dividing the organic ^{35}S to CP ratio in whole digesta (or feces) by the organic ^{35}S to CP ratio in bacteria isolated from the digesta (or feces).

Statistical Analysis. The experiment was carried out according to a 2x2 cross over design. The bacterial CP contribution to CP in digesta and feces between diets were analyzed using the GLM procedure of SAS (1988).

C. RESULTS AND DISCUSSION

Post mortem examinations indicated that their were no intestinal interconnections nor irregular intestinal adhesions.

Only small quantities (100-250 mg) of purified bacteria were collected during differential centrifugation and, as a result, the amount of purified bacteria available for some analyses was limiting. The amount of bacteria collected could be increased by the use of fresh digesta and feces combined with a greater number of collections per sample. The use of fresh digesta and feces would also increase the probability of collecting a representative bacterial pellet due to the lytic effect of freezing and thawing on bacteria (particularly the gram negative bacteria).

Incorporations of ^{35}S into the organic fractions of bacteria, digesta and feces were quite low (Table II.2). Sulfur-35 incorporations into the rumen samples described by Mathers and Miller (1980) were at least five fold higher. Increased ^{35}S incorporation rates into the bacteria would be desirable for future experiments to reduce the risk of the ^{35}S radioactivity reflecting background inorganic ^{35}S activity instead of the bacterial organically bound ^{35}S. A greater ^{35}S incorporation rate might be achieved by increasing the specific radioactivity in the infusate. Incorperation could be further increased via increasing the concentration of $^{32}SO_4^{2-}$ in the infusate, but only if the availability of inorgaic sulfur is limiting bacterial S incorperation.

The contribution of bacterial CP to digesta CP was significantly higher ($P < .05$) when the conventional compared to the purified diet was fed (Table II.3). The bacterial contribution to CP in feces also showed a trend ($P < .1$) towards larger values when the conventional diet was fed. The bacterial contribution to CP in ileal digesta of pigs fed both diets and in feces of pigs fed the conventional diet exceeded 100%. The value of 100% is the physiological for bacterial CP in total ileal digesta or fecal CP. The ^{35}S marker method in its present form can therefore not be used to estimate bacterial CP in either pig ileal digesta or fecal CP.

Speculating why the diets differed in their effects is of little value because the effects themselves are not physiologically meaningful. A more fundamental discussion relating to the origin of the bacterial CP% overestimations is perhaps more relevant. Overestimations may have resulted from either an elevated ^{35}S to CP ratio in the digesta (or feces) and/or a depressed ^{35}S to CP ratio in the bacterial isolates. Excess ^{35}S in the digesta (or feces) may have been in the form of $^{35}SO_4^{2-}$ that was not precipitated when $BaCl_2$ was added (i.e., if the sample matrix was able to bind free SO_4^{2-}). Mathers and Miller (1980) reported a precipitation rate of 98.6% for free $^{35}SO_4^{2-}$ from rumen digesta. In the present study, solubility product calculations indicated that the quantity of $BaCl_2$ added to the samples was far in excess of that required to precipitate any free SO_4^{2-}. If, however, small amounts of SO_4^{2-} from the infusate were able to escape precipitation from ileal digesta or feces, then bacterial CP overestimations would result. Two steps could be taken to increase $^{35}SO_4^{2-}$ removal from digestive samples in future experiments. First, inclusion and equilibration of $^{32}SO_4^{2-}$ in the oxidized hydrolyzed samples to allow dilution and exchange of $^{35}SO_4^{2-}$ prior to $BaCl_2$ addition may increase the efficiency of $^{35}SO_4^{2-}$ precipitation. Secondly, instead of using the crude ^{35}S-labelled organic fraction, an isolated marker like ^{35}S-methionine sulfone (Beever et al., 1974) could be used to reduce possible inorganic ^{35}S contamination.

The elevated ^{35}S activity in the digesta (or feces) may also have been organic in nature originating from endogenous and/or bacterial sources. Incomplete hydrolysis of endogenous sulfate esters (e.g., sulfated amino sugars, steroids and other lipids) would create artificially high organic ^{35}S activities in digesta (or feces). Apparent excessive endogenous labelling might be avoided if the turnover of SO_4^{2-} is slower in the endogenous than the bacterial pool. This would be accomplished by reducing the $Na_2{}^{35}SO_4$ infusion to 2 d while maintaining SO_4^{2-} availability for the bacteria with the 6 d $Na_2{}^{32}SO_4$ infusion. A shorter infusion of $Na_2{}^{35}SO_4$ would, however, prevent ^{35}S equilibration in the large intestine, and thus limit bacterial CP estimations to ileal digesta.

In addition to endogenous sources of organic ^{35}S, whole digesta and feces may have also contained confounding bacterial sources of organic ^{35}S. Bacteria in the purified pellets may have contained less organic ^{35}S per mg CP than bacteria in whole ileal digesta and/or feces (i.e., a nonrepresentative sample of bacteria may have been collected). In addition, organic ^{35}S in ileal digesta and/or feces may have originated from sulfur rich bacterial secretions (e.g., enzymes). The organic sulfur released from lysed bacteria may not have been as digestible as the non-sulfur organic matter. In addition, the lysed bacteria may have had a higher ^{35}S activity in their organic fraction than the whole bacteria. Higher organic ^{35}S activities from lysed bacteria could potentially be explained if bacteria synthesized in the upper digestive tract lysed before, and contained higher organic ^{35}S activities than bacteria growing close to the point of digestive sample collection. Indeed, differential labelling may have occurred if the concentration of infused SO_4^{2-} decreased along the digestive tract and/or small and large intestinal bacteria preferentially utilized ^{32}S rich dietary and/or endogenous materials to satisfy their sulfur requirements. To circumvent differential labelling interference in future studies, a change in the infusion site from the stomach to the proximal duodenum could be attempted. Blocking bacterial incorporation of ^{35}S in the stomach would be beneficial for two reasons. Upon reaching the distal ileum, bacteria originating from the stomach are more likely to lyse than intestinal bacteria due to their greater mean age and their exposure to the full wrath of the pigs digestive processes. The bacteria growing in the stomach can also potentially synthesize high specific activity ^{35}S organic material from $^{35}SO_4^{2-}$ due to the relative lack of free dietary or endogenous methionine and cysteine proximal to the small intestine.

Results from this investigation indicate that the method of Mathers and Miller (1980), for the ^{35}S marker determination of bacterial CP in rumen digesta, cannot be applied to similar measurements in swine feces and digesta. Possible explanations for the failure of this method have been outlined. From these speculations it is clear that future experimentation along the same line will only be fruitful upon substantial modification to the original method. In addition, because so many questions remain about the ^{35}S method, the use of a second bacterial marker (e.g., diaminopimelic acid) may be useful in future method developments.

TABLE II.1. COMPOSITION OF DIETS (%)

Ingredients	Conventional	Purified
Casein	0	18.9
Corn starch	0	61.9
Dextrose	0	10.0
Alfafloc[a]	0	3
Canola oil	0	3
Wheat	25.0	0
Barley	55.1	0
Soybean meal	16.0	0
Iodized salt	.4	.4
Dicalcium phosphate	1.5	.4
Calcium carbonate	1.0	1.4
Vitamin/mineral premix[b]	1.0	1.0
CP, as fed basis	16.5	16.5

[a] Lee Chemicals Ltd., Toronto, Ont., Canada

[b] Provided per kg diet: vitamin A, 1,300 IU; vitamin D, 150 IU; vitamin E, 11 IU; vitamin K, 2.0 mg; riboflavin, 2.2 mg; niacin, 12 mg; pantothenic acid, 11 mg; vitamin B_{12}, 11 μg; choline, 550 mg; thiamine, 1.1 mg; biotin, .1 mg; folic acid, .6 mg; Fe, 50 mg; Zn, 50 mg; Mn, 2 mg; Cu, 3 mg; I, 14 mg and Se .15 mg.

TABLE II.2. ORGANIC ^{35}S ACTIVITY IN BACTERIAL, ILEAL DIGESTA AND FECAL CP (BQ·MG^{-1} CP)[a]

Diet	Pig	Period	Il-D[b]	Il-B[b]	Feces	F-B[b]
Conventional	1	2	2.26	.51	3.33	2.89
	2	2	3.00	.56	3.27	2.43
	3	1	2.48	.42	9.04	7.17
	4	1	2.76	.50	7.10	5.74
Purified	1	1	1.91	.77	3.61	4.16
	2	1	2.01	.96	1.45	3.24
	3	2	1.69	1.52	2.67	3.37
	4	2	1.46	.44	1.05	4.37

[b] n = 1 (i.e., individual values)

[a] Il-D = ileal digesta; Il-B = bacteria in digesta; F-B = bacteria in feces.

TABLE II.3. PERCENT BACTERIAL CP IN TOTAL CP IN ILEAL DIGESTA AND FECES

	Diets			
Sample	Conventional	Purified	SEM[a]	P
Digesta	533	223	49.6	.04
Feces	124.3	58.8	14.0	.08

[a] Least square means, (n = 4).

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III. CELLULOSE CLEANUP AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF DL-DIAMINOPIMELIC ACID IN HYDROLYSATES OF PHYSIOLOGICAL SAMPLES[1]

A. INTRODUCTION

The partitioning of protein in a digesta sample into its fractions of origin is of considerable nutritional interest, particularly when the amino acid digestibilities from a feedstuff are to be determined. Most digesta samples contain a fraction of bacterial protein but this fraction cannot be estimated directly. Some of the bacteria can however be isolated from a sample of digesta via differential centrifugation and a compound that only the bacteria posses (a marker) can be used to quantify the amount of bacterial protein in the whole digesta sample. Simply, the bacterial protein content of a digesta sample is calculated by multiplying the concentration of the marker in the digesta by the protein to marker ratio found in a bacterial sample isolated from a different aliquot of the same sample of digesta.

Diaminopimelic acid (DAPA) is an amino acid that is often used as a marker to estimate the bacterial protein content in digesta samples. Several methods of analysis for

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[1] A version of this chapter has been published. Dugan, Sauer and Robinson 1989. Journal of Chromatography. 496:430.

DAPA have been developed (El-Shazly and Hungate, 1966; Hutton et al., 1971; Czerkawski, 1974; Mizik et al., 1978; Tunlid and Odham, 1983; Edols, 1985). These, however, either require a lot of time, labour, sample, and/or a high degree of technical expertise. A procedure enlisting the aid of high performance liquid chromatography (HPLC) has not been developed, probably due to problems with analytical column overloading, sample cleanup, and/or the lack of a good internal standard for complex physiological samples. The objective of this study was to develop a precise but relatively low labour input internal standard-based method for the cellulose clean-up, and HPLC analysis of DL-DAPA in hydrolysates of physiological samples.

B. EXPERIMENTAL PROCEDURE

Chemicals. DL-β-amino-n-butyric acid, DL-α,ϵ-diaminopimelic acid, diethylamine, Sigma aa-s-18 Amino Acid Standard, and o-phthaldialdehyde reagent solution were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium borate and HPLC grade water, acetone, methanol, 1-butanol, and tetrahydrofuran were purchased from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). CF 11 fibrous cellulose powder was obtained from Whatman Biosystems Ltd. (Maidstone, Kent, England).

Cellulose Columns. Cellulose columns were for single use only. Each column was prepared by firmly packing 1.0 g of fibrous cellulose powder into a glass wool plugged 5 mm I.D. Pasteur pipet.

Sample Type and Preparation. An attempt to determine the lower limit of DL-DAPA detection was carried out by using the Sigma aa-s-18 Amino Acid Standard (containing 17 amino acids) to simulate a 15% CP sample to which six levels of DL-DAPA were added. Each of these levels were assayed in triplicate. Variability of the assay was evaluated by analyzing two sources of physiological samples with four triplicated levels of DL-DAPA addition. The physiological samples included a rumen bacteria and a rumen digesta sample collected and pooled from several University of Alberta Dairy Research Unit dairy cow experiments in which a wide variety of diets were fed. Physiological samples were first hydrolysed (50 mg bacteria or 150 mg digesta; 2 mL 6 M HCl per 50 mg) in nitrogen purged screw cap culture tubes (16 h, 110 °C), and filtered through MicronSep 1 μm acetate membrane filters (M.S.I., Honeoye Falls, NY, U.S.A.). Samples, both physiological and simulated, were dried at 40 °C under vacuum with a Speed Vac Concentrator (Savant Instruments Inc. Farmingdale, NY, USA). The physiological samples were resuspended in water to provide levels of 6.7 mg and 20.0 mg of bacteria and digesta per mL with 0, 15, 45, and 75 nmol of DL-DAPA addition per mL. Simulated samples were resuspended in water to provide levels of 4, 8, 16, 32, 64, 128 nmol of DL-DAPA addition per mL. These resuspensions were then subjected to cellulose cleanup.

Cellulose Cleanup. Cellulose cleanup was pursued to both reduce the derivatized solute load going to HPLC and to selectively extract some of the amino acids (methionine, valine and phenylalanine) that migrate close to DL-DAPA during HPLC analysis. For each run, a cellulose column would first be conditioned with 4 mL solvent A (1-butanol:acetone:water:diethylamine; 65:10:10:6) (pH 11.4), then 100 μl of a resuspended sample would be applied to the column and eluted with a further 6.5 mL of solvent A. The DL-DAPA was then removed from the cellulose column with 7.0 mL of solvent B (acetone:water; 50:50). Both solvents A and B were introduced under pressure with a Harvard Apparatus Model No. 600-000 syringe infusion pump (Dover, MA., USA) running at 1.0 mL per min. The solvent B fraction containing the DL-DAPA was then freeze-dried, resuspended in 1.0 mL of water and taken for HPLC analysis. The cellulose columns and the solvent system used were modifications of a thin-layer chromatography procedure described by M. Brenner et al. (1969).

High Performance Liquid Chromatography. With a few modifications, analyses were completed according to the procedure described by Jones and Gilligan (1983) for the HPLC analysis of amino acids in physiological hydrolysates. Specifically, 75 μl of the resuspension from the cellulose cleanup was added to 25 μl of 25 nmol β-amino-n-butyric acid (internal standard) per mL 0.04 M sodium borate. The Varian 9090 Autosampler (Varian Instruments, Walnut Creek, CA, U.S.A.) was then employed to add and mix 50 μl of o-phthaldialdehyde reagent solution with the sample-internal standard mixture. The autosampler was then programmed to inject 10 μl of derivatized solution on to a Supelco guard column (50 mm length x 4.6 mm I.D) packed with LC-18 20-40 μm packing (Supelco. Inc., Bellefonte, PA, U.S.A.) to which a Supelcosil LC-18 3 μm particle size 150 x 4.6 mm I.D. HPLC column was attached. HPLC was carried out with a Varian Vista 5500 Liquid Chromatograph (Varian Instruments, Walnut Creek, CA, U.S.A.) equipped with a Varian 2070 Spectrofluorometer (Varian Instruments, Walnut Creek, CA, U.S.A.) set at 340 nm excitation and 450 nm emission. Compound retention times and peak heights were determined using a Hewlett-Packard series 3353 Laboratory Automation System (Avondale, PA, U.S.A.). Results were graphically plotted by a Fisher Recordall Series 5000 (Fisher Scientific Co., Edmonton, Alta., Canada). The two solvents, .1 M sodium acetate and methanol, were prepared according to Jones and Gilligan (1983). The gradient conditions used are described in table III.1. The DL-DAPA, under the conditions described, was found to elute at 19.0 min.

Statistical Analysis. Data to determine the lower limit of DL-DAPA detection were fitted to a quadratic regression model, with measured concentration of DL-DAPA as the dependant variable and addition levels of DL-DAPA as the independent variable (Steele and Torrie, 1980). The quadratic component of the model was not significant ($P > .35$). The data were, therefore, subjected to simple linear regression analyses to determine the recovery rate of DL-DAPA.

Data generated from DL-DAPA additions to physiological samples were analyzed to determine if sample type effected DAPA recovery rates using the GLM procedure of SAS (1988). The effects of DL-DAPA addition, physiological sample source and their interaction were included in the model. No significant difference ($P > .5$) was detected between sample types. Data from all additions were, therefore, used to calculate a pooled within run SE of recovery, a between run SE of recovery and an overall DL-DAPA recovery (Steele and Torrie, 1980).

C. RESULTS AND DISCUSSION

As shown in Figure III.1, cellulose cleanup quite effectively reduced the quantity of derivatized material injected on to the HPLC column yielding a cleaner chromatogram, particularly around the DL-DAPA peak. Figure III.1.a contains a DL-DAPA peak that is clearly quantifiable. Conditions used to generate this chromatogram were, however, close to ideal, which is not often the case when a physiological sample is analyzed. For this chromatogram a purified amino acid mixture was analyzed, a freshly packed guard column was used, and a new analytical column was employed.

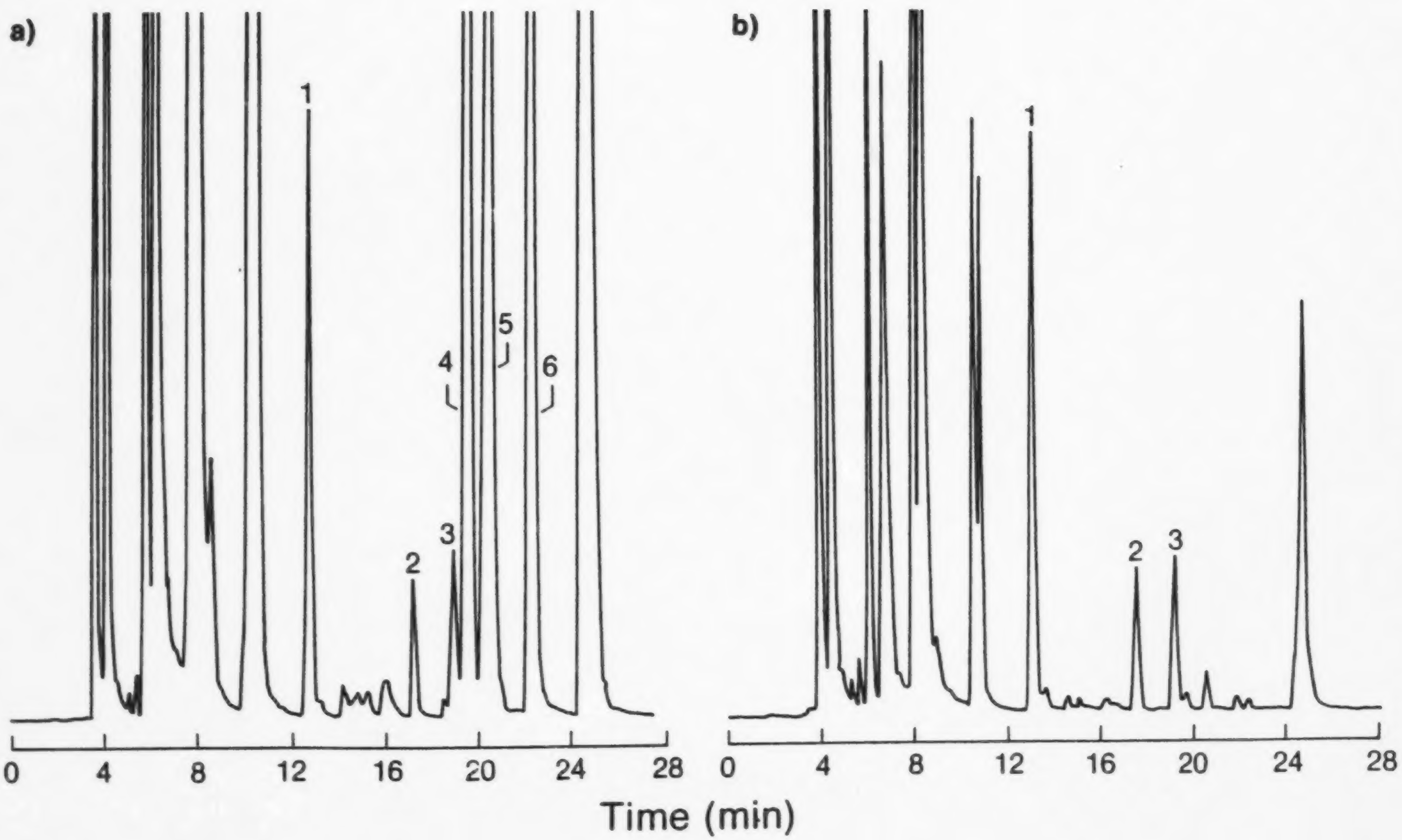
The lower limit of linear DL-DAPA detection was not reached, as judged by the non-significant ($P > .35$) quadratic component of the regression model. The method thus has the potential for linear detection of less than .06 μg DL-DAPA per mg dry sample, if 150 mg of rumen digesta were assayed. This translates to an on-column injection and detection of approximately 3.3 pmol of DL-DAPA. The recovery of DL-DAPA from the simulated 14% CP sample, calculated using simple linear regression analysis, was 96.76 ± .44%. The DL-DAPA recoveries for the bacterial samples were not significantly different from those of the digesta samples ($P > .5$). Combined, the bacteria and digesta gave an average recovery of 96.20% with a within run pooled SE of ± 2.14% and a between run SE of ± .49%. These results indicate that the method is analytically precise and would be useful where minute quantities of DL-DAPA need to be measured, particularly when sample cleanup is required. In addition, as the recoveries of the two physiological samples were not different, separate recovery curves for each sample to estimate total DL-DAPA contents may not be needed.

TABLE III.1. HPLC GRADIENT CONDITIONS[a]

Time (min)	.1 M Sodium Acetate, %
0.0	100
0.1	70
21.7	50
21.8	0
24.2	0
24.7	100

[a] Flow rate: 1.1 mL/min; temp: 21 °C; run time: 28 min.

Figure III.1. Chromatograms of the simulated 15% CP sample with a 13.5 nmol DL-DAPA addition per mL before cellulose cleanup: (a) without cellulose cleanup and (b) with cellulose cleanup. Peaks: 1 = DL-β-amino-n-butyric acid, 2 = LL and DD-DAPA (included in the DL-α,ϵ-diaminopimelic acid standard), 3 = DL-DAPA, 4 = methionine, 5 = valine, 6 = phenylalanine.


a)
1
2
3
4
5
6
b)
1
2
3
0
4
8
12
16
20
24
28
Time (min)

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IV. A VORTEX VACUUM EVAPORATOR FOR SAMPLES IN TEST TUBES[2]

A. INTRODUCTION

The trend in animal science towards micro-analyses has enabled many analyses to be performed on a test tube scale. Analytical schemes often require one or more drying stages. Frequently used techniques for drying samples include evaporation while heating under a stream of dry gas, evaporation in a vacuum oven, roto-evaporation, freeze drying, vacuum centrifuge drying, and drying under vacuum while vortexing in a heated sample block.

Drying under a stream of gas is both slow and expensive. Large volumes of gas are used, and added labour and equipment times are needed during extended drying periods. Vacuum oven drying is slow, and neither the vacuum oven nor the roto-evaporator are practical for test tube scale drying due to sample loss and/or contamination caused by bumping (erratic boiling when too much vacuum is applied). Freeze dryers and vacuum centrifuges are expensive, slow per tube dried, unless large numbers of samples are dried at once and subject to damage when corrosive volatiles are present. The vortexing block dryer is also expensive and subject to damage by corrosive volatiles but avoids slow drying and bumping by generating a sample vortex while drying. It is thus apparent that test tubes are not ideally suited vessels for drying samples and this prompted our lab to develop a rapid, trouble free and inexpensive drying unit for samples up to 5 mL original volume.

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[2] A version of this chapter has been published. Dugan, Sauer and Fenton 1992. Canadian Journal of Animal Science. 72:189.

B. EXPERIMENTAL PROCEDURE

The design of the vortex vacuum evaporator is relatively simple (Figure IV.1). It employs a vortex to increase sample surface area and reduce bumping while remaining inexpensive and corrosion resistant. Design specifications listed in Figure IV.1 are not rigid. The diagram merely represents a working model presently in use in our laboratory for 5 mL samples. A sample to be dried is placed in a screw capped test tube, and the tube is immersed in a water jacket to provide heat for evaporation. Water jacket heating was chosen over other methods of heating because it was thought to be able to yield the highest rate of heat transfer to the sample, and thus the fastest rate of drying. In addition, the temperature of a water jacket can start low, and then be rapidly adjusted to the optimal drying temperature of the sample. Rapid jacket temperature change can save time because after an initial critical bumping phase, sample temperature and drying rate can usually be increased quickly without problem. The sample tube is sealed in the water jacket with a screw cap and rubber O-ring. The jacket is made by adding glass side arms to a screw capped test tube that is both shorter and wider than the sample tube. A vortex is generated in the sample tube using a Vortex Genie™ (Scientific Industries, Bohemia, N.Y., U.S.A.). To start the drying process, a vacuum is applied to the sample tube through a modified cap using a Duo Seal™ vacuum pump (Welsh Scientific Co., Chicago, Ill., U.S.A.). The vacuum pump is protected with an in-line liquid nitrogen cold trap to catch evaporated materials originating from the sample. Only inexpensive and relatively inert components are located upstream from the cold trap making the vortex vacuum evaporator ideal for work with corrosives.

To demonstrate the performance of the vortex vacuum evaporator it was tested against two high speed alternatives, the Speed Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.) and the Virtis 50 SRC-5 freeze dryer (The Virtis Company, Gardiner, NY, U.S.A.). The water jacket temperature of the vortex evaporator was set at 40 °C. The temperatures of the

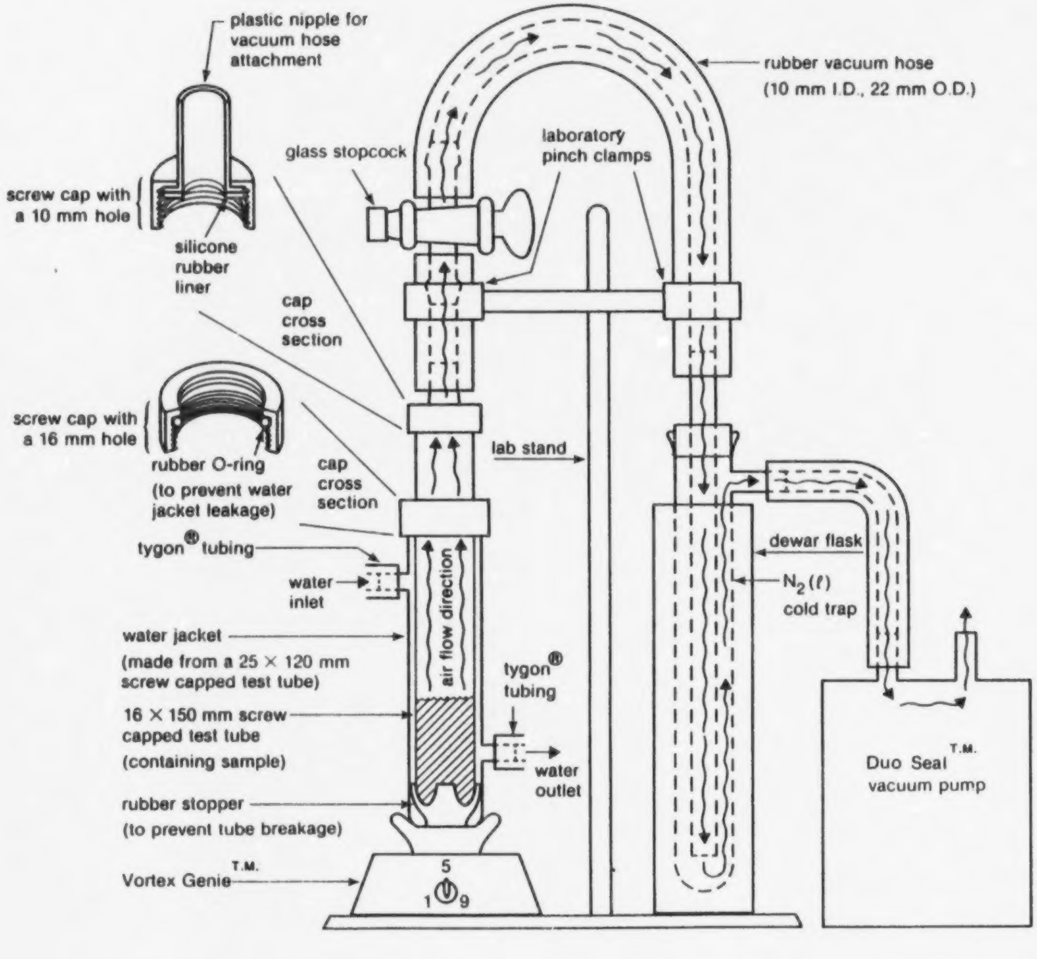
Speed Vac centrifuge and cold trap were 40 °C and -60 °C respectively. The Speed Vac concentrator was fitted with the same Duo Seal™ vacuum pump as used with the vortex vacuum evaporator. The freeze dryer shelf and condenser temperatures were set at 20 °C and -60 °C respectively. A 20 °C shelf temperature was used, instead of the 40 °C sample temperature of the other dryers to prevent thawing during sublimation. The three machines were compared on the basis of the time taken to dry ten 5 mL water samples. The tubes dried on the vortex vacuum evaporator were done consecutively with the evaporator set up and disassembly times included in the total drying time. The tubes dried in the Speed Vac concentrator and the Virtis 50 SRC-5 freeze dryer were dried in single batches with the final drying times extrapolated from 5 h drying rates.

C. RESULTS AND DISCUSSION

The vortex vacuum evaporator took 1.17 h to dry the ten tubes at 42.6 mL h^{-1}. This compares favourably to the 7.11 h for the Speed Vac at 7.03 mL h^{-1} and 11.96 h for the freeze dryer at 4.18 mL h^{-1}. These results indicate the vortex vacuum evaporator is well suited for the rapid drying of limited numbers of small samples. Our laboratory has found it particularly useful in method development where several sets of a few tubes are dried over a number of days.

The vortex vacuum evaporator is similar to a vortexing block dryer, for example the Evapotec™ (Haake Buchler Instruments, Saddlebrook, NJ, U.S.A.), but the vortex vacuum evaporators thermo-conductive characteristics are superior and it is relatively corrosion resistant. The vortex vacuum evaporator is also an extremely economical piece of equipment. Assuming a lab has a Dewar flask, vortex generator and a vacuum pump, the vortex vacuum evaporator costs approximately $11.40 to manufacture versus the purchase cost of $6359.33 for an Evapotec™ (also without a cold trap or vacuum pump). The only draw back of the vortex vacuum evaporator appears to be its high labour requirement (the evaporator while operating requires constant attention). However, the overall labour requirement per tube could be substantially reduced by attaching a series of vortexing units to the same vacuum source. Thus the vortex vacuum evaporator, in many circumstances, is a very practical alternative to other high speed drying systems.

Figure IV.1. A heat assisted vortex vacuum evaporator for small liquid samples


plastic nipple for vacuum hose attachment
screw cap with a 10 mm hole
silicone rubber liner
glass stopcock
laboratory pinch clamps
rubber vacuum hose (10 mm I.D., 22 mm O.D.)
cap cross section
screw cap with a 16 mm hole
rubber O-ring (to prevent water jacket leakage)
cap cross section
lab stand
tygon® tubing
water inlet
air flow direction
water jacket (made from a 25 × 120 mm screw capped test tube)
tygon® tubing
16 × 150 mm screw capped test tube (containing sample)
water outlet
rubber stopper (to prevent tube breakage)
Vortex Genie T.M.
5
1
9
dewar flask
$N_2(\ell)$ cold trap
Duo Seal T.M. vacuum pump

V. DETERMINATION OF THE BACTERIAL CONTRIBUTION TO AMINO ACIDS IN PIG ILEAL DIGESTA USING A ^{35}S AND A DAPA MARKER TECHNIQUE[3]

A. INTRODUCTION

Currently, protein sources for swine are often ranked on the basis of their supply of digestible amino acids, as determined with the ileal analysis method. Until recently, the content and activities of bacteria proximal to the large intestine have been dismissed as being of little importance in nutritional terms (Hungate, 1984; Savage, 1986). However, evidence to the contrary is becoming increasingly evident in the literature. Inclusion of subtheraputic levels of antibiotics in swine diets improved growth performance (e.g., Yen et al., 1987) and reduced intestinal bacterial ammonia production (Yen et al., 1990), bacterial primary amine production, apparent amino acid absorption efficiency (Dierick et al., 1986), and the level of lumenal adenosine triphosphate presumed to be of bacterial origin (Jensen, 1988).

An initial step in examining the influence that small intestinal bacteria have on amino acid digestibility is by determining the bacterial contribution to the total protein found in ileal digesta. Of the ileal digesta crude protein (CP) and total amino acids, 20 to 35% have been estimated to be of bacterial origin when pigs were fed cereal based diets when using diaminopimelic acid (DAPA) for a bacterial marker (Dierick et al.(1983) and Poppe et al. (1983) measured the bacterial CP; Drochner (1984) measured bacterial amino acids). Drochner (1984), in the only study in which descriptive statistics were provided, reported a large variation in the bacterial contribution to amino acids in ileal digesta. To determine if the variability of the bacterial amino acid estimations were due to the use of the DAPA marker per se, an initial study was conducted (Chapter II) using $Na_2{}^{35}SO_4$ to mark the organic matter of bacteria in pig ileal digesta and feces. The ^{35}S technique was adapted from the method of Mathers and Miller (1980) used for determining the bacterial contribution to CP in sheep rumen digesta. Results reported in Chapter II were not realistic due to the estimation of bacterial percentages of CP in ileal digesta CP and fecal CP exceeding 100% (in some cases up to 500%).

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[3] A version of this article has been submitted for publication. Dugan, Sauer and Lien 1992. Journal of Animal Physiology and Animal Nutrition.

The objectives of the present study were to determine the bacterial contribution to amino acids in swine ileal digesta by using both ^{35}S and DAPA as bacterial markers. To improve upon previous methodology, the ^{35}S technique originally used (Chapter II) was modified by increasing the

$^{35}SO_4^{2-}$ specific radioactivity and the SO_4^{2-} concentration in the infusate, changing the site of infusion from the stomach to the duodenum, and by isolating a specific sulfur containing marker fraction (methionine sulfone) from both bacteria and digesta. DAPA was employed as an additional marker to enable comparison of the two markers and reduce the likelihood of error when a single marker is used.

B. EXPERIMENTAL PROCEDURE

Two gilts (Lacombe x Yorkshire), with an average initial body weight of 45 kg, were surgically fitted with a simple T-cannula in both the proximal duodenum and distal ileum. The design of the ileal cannula used was initially described by Sauer et al. (1983) and later modified according to de Lange et al. (1989). The duodenal cannula was technically similar to the re-entrant arm of the pancreatic cannula described by Hee et al. (1985). The surgical technique of Sauer et al. (1983) was adapted for the insertion of the ileal cannula; the duodenal cannula was inserted using the technique described by Hee et al. (1985) for inserting the re-entrant pancreatic cannula arm. The gilts were fasted for a 24 h period prior to surgery. One hour before surgery the animals were sedated with a 1 mL intramuscular injection of Atravet (Ayerst Laboratories, Montreal, Que., Can.). After surgery, the gilts were placed in individual stainless steel metabolic crates in a barn maintained at 20 °C. The animals were allowed a 10 d recovery period during which time an 18% CP starter diet (Sauer *et al.*, 1983) was fed twice daily at 0600 and 1800 h. The diet was introduced gradually the day after surgery from 50 g per feeding to *ad libitum* by the fifth day. To reduce post-surgical pain, Torbugesic (Ayerst Laboratories, Montreal, Que., Can.) was added to the feed (1.2 mg per kg body weight).

The experimental period lasted 13 d. During the experiment, the animals were fed a 16.5% CP conventional grower diet (Chapter II). Chromic oxide was included in the diet at a level of .4% to enable digestibility measurements. The diet was fed four times daily at 0600, 1200, 1800 and 2400, 550 g per feeding. Water was available at all times from a low pressure drinking nozzle. During each day of the experiment a continuous (24 h) minfusion of 512 mL of an aqueous solution containing 4.35 g of NaCl and 1 g of Na_2SO_4 was made into the duodenum of each pig. On days 12 and 13, 55.5 MBq of $Na_2{}^{35}SO_4$ (ICN Biomedicals, Irvine, CA, U.S.A.) were added to the infusate for each animal. During the last 12 h of infusion on day 13, ileal digesta samples were collected for two 6 h periods into flexible polyethylene tubing (1.5 m length, 4 cm width). One end of the tubing was connected to the cannula, the other end sealed and submerged in ice water. Outflowing digesta were immediately shunted into tubing submerged in ice water to reduce the rate of bacterial metabolism.

Bacterial Separations. After each collection period ice cooled ileal digesta were immediately taken for bacterial separations. The samples were mixed and six 100 mL subsamples of digesta were taken. Each subsample was added to 800 mL of 4 °C diluent containing 8.5 $g \cdot L^{-1}$ NaCl, 6 $g \cdot L^{-1}$ $MgSO_4$ and 2 mL of Triton-x-100. The bacterial metabolic activity was decreased by keeping the digesta at a low temperature instead of reducing the pH as described in Chapter II. Triton-x-100 was added to aid in the separation of loosely bound bacteria from feed particles. Magnesium sulfate was added to stabilize the gram-negative bacterial cell membranes, as recommended by Gerhardt (1981). The suspensions were mixed for 40 min using Nuova II stir plates (Thermolyne Corp., Dubuque, Iowa, U.S.A.) set at speed seven. Feed particles were removed from suspension by centrifuging twice at 200 *g* for 5 min. Each centrifugation was followed by filtration through several layers of glass wool. After low speed centrifugation, the supernatant was examined microscopically (with and without gram-staining) and found to be relatively free of feed particles. Bacteria in the supernatant were then pelleted by centrifuging at 5100 *g* for 10 min. Bacterial pellets were washed with 500 mL of 4 °C diluent, and centrifuged again (5100 *g* for 10 min) and finally collected for subsequent analyses. The additional washings carried out previously (Chapter II) were abandoned to minimize bacterial lysis. The water soluble materials were estimated to have already undergone a 12,000 fold dilution. Both high and low speed centrifuging were carried out at 4 °C in a Beckman J-6B/P Centrifuge (Beckman, Palo Alto, CA, U.S.A.).

The 5100 *g* centrifugation used in this study was reduced from the 9100 *g* used previously (Chapter II) to ensure that whole and few bacterial fragments, particularly cell walls, would be collected in the bacterial pellet (Schnaitman, 1981). This precaution was taken because DAPA is present in high concentrations in bacterial cell walls (i.e. a disproportionately high cell wall content in the pellet would result in an overestimation of the DAPA to bacterial amino acid ratio in ileal digesta). Bacterial amino acid calculations (outlined in a following section), therefore, represent the maximum bacterial contribution to amino acids in ileal digesta.

Chemical Analyses. Samples of digesta, bacteria and diet were freeze dried and ground through a 1 mm screen prior to analyses. Dry matter determinations were carried out on feed and digesta (AOAC, 1984). Chromic oxide concentrations in feed and digesta were determined according to the procedure of Fenton and Fenton (1979). Three methods were used to analyze the amino acid content in bacteria, digesta and the diet including a procedure for the sulfur containing amino acids , a procedure for DL-DAPA and a procedure for the amino acids listed in Table V.5 excluding the sulfur containing amino acids (DL-DAPA and the amino acids listed in Table V.5 were analyzed using aliquotes from the same hydrolysate, and seperate hydrolysates were used for the sulfur containing amino acids). To prepare the samples for DL-DAPA and Table V.5 amino acids (excluding the sulfur containing amino acids), either 60 mg of bacteria, 200 mg of digesta or 100 mg of the diet were taken (in duplicate) and hydrolyzed in $N_{2(g)}$ purged screw capped (13 x 100 mm) culture tubes for 20 h at 110 °C in 6 M HCl. The amino acids listed in Table V.5 (excluding the sulfur containing amino acids) were analyzed according to the procedures described by Jones and Gilligan (1983) using the high performance liquid chromatography (HPLC) columns and equipment previously described in Chapter III. DL-DAPA analysis was carried out using the method described in Chapter III. The DL-DAPA concentrations were calculated based on external standards instead of the internal standard (β-amino-butyric acid) due to the presence of an unknown compound which co-eluted with the internal standard. Each hydrolysate was analyzed in quadruplicate for its DL-DAPA content. The solvent gradient used for the HPLC analysis of DL-DAPA (Table V.1) was altered from that used previously (Chapter III) in order to enable separation of DL-DAPA from interfering compounds. Under these gradient conditions DL-

DAPA was found to elute in 27.4 min.

The sulfur containing amino acids were quantified and prepared for scintillation counting using a modified combination of the methods described by Beever et al. (1974), Mathers and Miller (1980) and Jones and Gilligan (1983). Specifically, either 60 mg of bacteria, 200 mg of digesta or 100 mg of diet were oxidized in duplicate with performic acid (4 mL for 16 h at 4°C) in closed screw capped test tubes (16 x 150 mm). Performic acid was prepared as described by Mathers and Miller (1980). Oxidation was halted with the addition of .6 mL of 48% HBr. The volumes of acids used per mg of sample were less than used by Mathers and Miller (1980) and Beever et al. (1974). Preliminary studies using smaller acid volumes indicated oxidation would go to completion in the experimental samples. After HBr addition, the samples were dried using the dryer described in Chapter IV. The dry samples were then hydrolyzed (using the hydrolysis procedure noted above for the other procedures) and re-dried using a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY, U.S.A.) and subsequently resuspended in 6 mL of water. From this resuspension, .5 mL were taken (in duplicate) for methionine sulfone and cysteic acid analysis. The analysis was completed after minor modification to the method described by Jones and Gilligan (1983). Modifications consisted of a solvent gradient change (Table V.2) to optimize the resolution of peaks of interest, the use of the internal standard L-α-amino-β-guanidino-propionic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) and a change in the acetate solvent composition (120 mL methanol instead of 180 mL and a pH adjustment from 7.2 to 6.75).

The remaining 5 mL of the oxidized hydrolysed samples were subjected to cation exchange clean-up to separate methionine sulfone from the other ^{35}S containing compounds (e.g., sulfate and cysteic acid). This was accomplished by loading the remaining 5 mL onto a 6 x 180 mm glass column with a glass wool plug containing Dowex-50 hydrogen form resin (8x cross linked, 100 to 200 dry mesh; Sigma Chemical Co., St. Louis, MO, U.S.A.). Sulfur containing compounds other than methionine sulfone were eluted with 11 mL of water followed by 5 mL of 2.5 M NH_4OH. The methionine sulfone fraction was then eluted with 8 mL of 2.5 M NH_4OH. The eluants were dried in a Speed Vac concentrator and resuspended in 5 mL of water. From each resuspension, .5 mL was taken in duplicate and analyzed to determine the methionine sulfone recovery and to check for complete removal of cysteic acid. To ensure $^{35}SO_4^{2-}$ removal from the remaining 4 mL of sample, .25 mL of .5 M $Na_2{}^{32}SO_4$ was added, mixed and left to equilibrate with the samples for 15 min. Then .25 mL of 1.0 M $BaCl_2$ was added, mixed and left to stand for 15 min. The $BaSO_{4\,(s)}$ was then removed from suspension via centrifugation at 2300 *g* for 15 min. The supernatants were then prepared for scintillation counting by adding and mixing 2 mL of sample (in duplicate) with 12 mL of Ecolite™ Scintillation Counting Fluid (INC Biomedicals Inc., Irvine, CA, U.S.A.). Scintillation counting was then carried out using a Searl Mark III 6880 Liquid Scintillation System (Searle Analytical Inc., Des Plaines, Ill, U.S.A.). The ^{35}S activity (Bq) in the samples was determined after corrections for background and counting efficiency were made.

Bacterial Amino Acid Calculations. The calculation for determining the bacterial percentages of amino acids in ileal digesta amino acids were similar to the calculations detailed in Chapter II, except that the calculations were carried out using concentrations of individual amino acids instead of CP. In addition, both ^{35}S-methionine sulfone and DAPA were used as markers.

Statistical Analysis. The amino acid compositions of bacteria and digesta (corrected for its bacterial content) were analyzed according to the GLM procedures of SAS (1988) using a repeated measures ANOVA. The repeated measures were the percentages of amino acids in bacteria and digesta in two pigs over two time periods. Simple regression analyses, regressing apparent amino acid digestibility coefficients (%) versus the bacterial amino acid contents of whole digesta (%), were also conducted. Regression coefficients (β_1) were analyzed using the t-test according to Steele and

Torrie (1980).

C. RESULTS AND DISCUSSION

After the experiment, the pigs were sacrificed and post-mortem examinations conducted to inspect the cannulation sites for abnormalities. Neither intestinal interconnections nor irregular intestinal adhesions were observed.

The bacterial contribution to total amino acids in ileal digesta, estimated using the ^{35}S-methionine sulfone marker, exceeded the physiological limit (100%), while estimates based on DAPA fell within the physiologically range (< 100%) (Table V.3).

The ^{35}S results were likely confounded, as in the previous studies (Chapter II), by either bacterial and/or endogenous sources. Either bacterial and/or endogenous compounds elevated the ^{35}S activity in digesta resulting in an overestimation of the bacterial contribution to amino acids in ileal digesta. The present ^{35}S results appear closer to the upper limit for the bacterial percentage of total ileal digesta amino acids arising from bacteria (100%) than the results obtained in Chapter II. Modifications to the method described in Chapter II were insufficient to rectify the overestimations. Future ^{35}S experiments to provide specific explanations for the results obtained should concentrate on the isolation of a purified marker fraction instead of the fractions described by Beever et al. (1974) and Mathers and Miller (1980). An attempt to isolate the methane-thiol group from methionine was made by reacting methionine with cyanogen bromide and extracting the methylthiocyanate product into a non-polar phase. This technique for specific marker isolation was based on the method of Paul (1977) for the determination of methionine in field peas. These attempts were unsuccessful, probably due to the use of free instead of protein bound methionine as the methane-thiol source (i.e., free methionine is likely not as reactive as protein bound methionine).

The average concentrations of DL-DAPA in ileal digesta and isolated bacteria were .07 ug and .4 ug per mg respectively. The contents of DL-DAPA in ileal digesta and isolated bacteria were approximately seven and five fold less than the total DAPA concentrations reported in pig feces and fecal bacteria by Laplace et al (1985) . These differences may be attributed to the measurement of only DL-DAPA in the present experiment, and the affect of diet and digestive tract location on the bacterial populations composition and number. Concentrations of DL-DAPA in bacterial samples in the present experiment may have been also low due to their relatively high concentration of $NaCl_{(s)}$ (normal saline was used to transfer bacteria from centrifuge bottles to vials for drying). Elevated between pig variation in the bacteria and digesta content of DL-DAPA was also noted. Pig one digesta and bacteria contained approximately 4 fold less DL-DAPA than pig two. Elevated between pig variation was also noted by Laplace et al. (1985). This variation indicates that the bacterial population may differ between pigs, at the level of the ileum, when feeding the same diet.

The bacterial contribution to amino acids in ileal digesta (29.4 ± 3.6%), when measured using the DAPA marker technique, was within the range of results (20-35%) reported in previous studies with pigs fed cereal based diets (Dierick et al., 1983; Poppe et al., 1983; Drochner, 1984). In the present study, although limited sample numbers prevented statistically meaningful comparisons, bacteria appeared to consistently contribute to total amino acids in ileal digesta within pig over time. However, a rather large difference (7 percentage units) was noted between pigs. Large variations (33 ± 8.3 SD) between pigs were also reported by Drochner (1984) in studies in which a corn-soybean meal diet was fed. The large variation in the bacterial content between pigs may be due to a relatively small change in the apparent amino acid digestibilities (Table V.4). For example, if one additional percentage unit of dietary amino acids was not absorbed but was assimilated by bacteria, assuming bacteria initially accounted for 30% of ileal digesta amino acids and there was an average apparent amino acid digestibility of 80%, then the bacterial contribution to amino acids in the ileal digesta would increase by 3.3%. Elevated between pig variation may also be due to differences in the types of bacteria found in the digestive tracts of different pigs fed the same diet (as indicated by the different DL-DAPA concentrations in the bacterial and digeata samples).

Differences between the amino acid composition of bacteria and the undigested non-bacterial residuals were noted (Table V.5)(even when the make-up of the populations of bacteria apparently differed between pigs). Percentages of five of the 16 amino acids analyzed (isoleucine, aspartic acid, glycine, cysteine and tyrosine) were significantly ($P < .05$) different. This would indicate that the amino acid composition of protein in ileal digesta may be influenced by its bacterial amino acid content. However, this influence will be limited due to the relatively low (29.4%) bacterial amino acid contribution to the total amino acids in the ileal digesta.

The significantly lower ($P < .05$) glycine content in bacteria compared to the undigested endogenous and dietary residuals is of particular interest. The glycine content of endogenous protein in digesta collected from the distal ileum was found to be quite high relative to the other amino acids (e.g., de Lange et al., 1989). The lower glycine content of bacteria would therefore suggest that the bacterial fraction was relatively free of contamination of endogenous sources.

To determine if subtle changes in apparent ileal amino acid digestibilities might be accounted for by changes in the ileal bacterial amino acid content, apparent ileal amino acid digestibility coefficients (Y) were regressed versus the percentages of dietary amino acids found in ileal bacteria (X) (% dietary amino acids in ileal bacteria = % apparently undigested amino acids x % bacterial amino acids contained in the undigested amino acids). As might be expected, the slopes (β_1) for all the regression lines were negative, with four of the slopes being significant (Table V.6). The amino acids with significant slopes included threonine (-2.45), alanine (-2.15), tyrosine (-1.26) and lysine (-1.76). The negative slopes not only indicate an assimilation of amino acids by bacteria, but because the majority of the slopes (11 out of 16) are less than minus one, reduced digestibilities might partly be explained by increased digesta levels of non-bacterial components (either increased endogenous and/or undigested dietary residuals).

As noted previously, the bacterial contribution to total amino acids in ileal digesta was within a reasonable range when using the DAPA marker technique. It must, however, be stressed that both the DAPA and ^{35}S marker techniques are based on the assumption that a representative purified bacteria pellet is collected. The results obtained with the ^{35}S technique exceeded the maximum possible physiological range. However, this does not mean that the results obtained using this technique are not important. In actuality, the lack of agreement between the two marker systems puts both techniques into question. Clearly, further investigations into determining the bacterial contribution to the amino acids in ileal digesta are warrented.

TABLE V.1. HPLC SOLVENT GRADIENT FOR DAPA ANALYSIS

Time, min[a]	% Acetate Solvent
0	100
.1	69
38.3	59
38.4	0
41.4	0
41.5	100

[a] Total run time: 42 min.

TABLE V.2. HPLC SOLVENT GRADIENT FOR ANALYSIS OF SULFUR CONTAINING AMINO ACIDS

Time, min[a]	% Acetate Solvent
0	100
3	95
3.1	86
29.5	83
29.6	0
31.6	0
32.6	100

[a] Total run time: 33 min.

TABLE V.3. PERCENT OF TOTAL ILEAL AMINO ACIDS ARISING FROM BACTERIA

Pig	Period	^{35}S-Methionine Sulfone	DAPA
1	1	288.0	32.6
1	2	255.0	33.1
2	1	95.5	26.7
2	2	107.5	25.1
average[a]		190.6 ± 85.6	29.4 ± 3.6

[a] Means ± between pig standard error.

TABLE V.4. PARTITIONING OF THE TOTAL DIETARY AMINO ACIDS (%)[a]

	Pig 1			Pig 2		
Period	D	B	R	D	B	R
1	83.7	5.3	11.0	82.8	4.6	12.6
2	81.8	6.0	12.2	84.7	3.9	11.5

[a] D = digested; B =ileal bacteria; R = ileally undigested endogenous and dietary residuals.

TABLE V.5. AMINO ACID COMPOSITION OF ILEAL BACTERIA (B) AND ILEALLY UNDIGESTED ENDOGENOUS AND DIETARY RESIDUALS (R) EXPRESSED AS A PERCENTAGE OF TOTAL ILEAL AMINO ACIDS

Amino acid	B	R	SE[a]
Indispensable			
Arginine	7.34	5.48	.505
Histidine	2.62	2.78	.056
Isoleucine	6.46	5.37	.032*
Leucine	9.76	9.37	.381
Lysine	7.95	5.76	.720
Methionine	1.16	1.69	.195
Phenylalanine	6.62	6.12	.397
Threonine	4.84	6.44	.428
Valine	6.49	6.75	.241
Dispensable			
Alanine	6.42	6.13	.648
Asx	14.49	13.10	.046*
Glx	9.51	11.28	.566
Glycine	4.83	7.67	.062*
Cysteine	1.85	4.56	.057*
Serine	5.07	4.69	.500
Tyrosine	4.58	2.72	.173*

[a] Pooled standard error of the mean (n = 4).
*(P < .05)

TABLE V.6. REGRESSION COEFFICIENTS (β_1) FOR INDIVIDUAL AMINO ACIDS (APPARENT ILEAL AMINO ACID DIGESTIBILITY (%)(Y) REGRESSED VERSUS THE ILEAL BACTERIAL CONTENT OF DIETARY AMINO ACID (%)(X))

Amino acid	β_1	SEa
Indispensable		
Arginine	-.26	.46
Histidine	-1.08	.55
Isoleucine	-1.01	.48
Leucine	-.81	.52
Lysine	-1.76	.40*
Methionine	-3.01	1.98
Phenylalanine	-.70	.44
Threonine	-2.46	.32*
Valine	-1.59	.49
Dispensable		
Alanine	-2.45	.07*
Aspartic acid	-1.84	.44
Glutamic acid	-.12	.54
Glycine	-1.28	1.11
Cysteine	-1.43	1.84
Serine	-.35	.46
Tyrosine	-1.26	.28*

[a] Standard error of β_1.
*($P < .05$)

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VI. ION PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF DIAMINOPIMELIC ACID IN HYDROLYSATES OF PHYSIOLOGICAL SAMPLES[4]

A. INTRODUCTION

The nutritive value of a protein source is, in part, dependent on its digestible amino acid supply which can be determined with the ileal analysis method. The ileal analysis method measures the difference between the amount of each amino acid consumed and recovered in digesta collected from the distal ileum (Sauer and Ozimek, 1986). However, the measurement of the digestible amino acid supply with this method is confounded due to the presence of bacteria in the digestive tract. Bacteria can utilize dietary amino acids in addition to synthesizing their own amino acids. In terms of nutritional evaluation it is therefore important to estimate the amount of bacterial amino acids in ileal digesta.

Diaminopimelic acid (DAPA) is an amino acid found almost exclusively in gram-positive bacteria. It is therefore used as a marker amino acid to determine how much bacterial protein is present in a digesta sample. The amount of bacterial protein in a digesta sample is calculated by multiplying the DAPA concentration in the digesta sample by the protein to DAPA ratio found in purified bacteria collected from the digesta sample. It is necessary to determine the amount of bacterial protein using a bacterial marker because it is impossible to quantitatively separate all the bacteria from a particular digesta sample.

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[4] A version of this chapter has been accepted for publication. Dugan, Sauer, Fenton and Lien 1992. Journal of Chromatography

Early methods for DAPA analysis in physiological samples (El-Shazly and Hungate, 1966; Hutton et al., 1971; Czerkawski, 1974; Mizik et al., 1978 and Edols, 1985) relied on ion-exchange chromatography followed by post-column ninhydrin reaction and detection of the DAPA-ninhydrin derivative. These methods, although reliable, have given way to more economical and sensitive high-performance liquid chromatography (HPLC) methods (Chapter III and Webster et al., 1989) using pre-column o-phthaldialdehyde (OPA) derivatization of DAPA. Some problems with the HPLC methods have, however, been found. First, obtaining adequate resolution of the two DAPA peaks can be difficult because the DAPA derivatives elute slower than surrounding derivatives with the polar solvent (.1 M acetate) and then faster with the non-polar solvent (methanol). In addition, even after using the cellulose clean-up procedure described in Chapter III, measurement of both DAPA peaks is often difficult when DAPA concentrations are close to levels of residual co-elutants.

The DAPA-OPA derivatives possess two carboxyl groups whereas most other amino acid-OPA derivatives have one. Ion pairing with a cationic detergent should thus enable the preferential retention of the DAPA-OPA derivatives on a reverse-phase column. The first objective of this study was, therefore, to determine if ion pairing with hexadecyl-trimethyl-ammonium bromide (HTMA) could be used to facilitate DAPA analysis. The second objective was to find a suitable internal standard for use with physiological samples, and then finally to make the method as simple and inexpensive as possible by avoiding any extraneous cleanup, filtering, transferring or drying stages.

B. EXPERIMENTAL PROCEDURE

Chemicals. Sodium borate, potassium borate, sodium acetate, HPLC grade water, HPLC grade methanol and glacial acetic acid were purchased from BDH (Toronto, Ont., Canada). DL-α-amino-caprylic acid, hexadecyl-trimethyl-ammonium bromide, o-phthaldialdehyde reagent solution (OPA), and DL-α,ε-diaminopimelic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

DL-α,ε-DAPA, under chromatographic conditions defined later, elutes as two separate peaks. The first peak is assumed to contain the DD and LL-DAPA enantiomers, and the second peak is assumed to contain the more naturally abundant enantiomer, DL-DAPA . The first DAPA peak will therefore be referred to as DD,LL-DAPA and the second as DL-DAPA. The DL-α,ϵ-DAPA used in these experiments contained a 50:50 mixture of DD,LL-DAPA and DL-DAPA.

Sample type and preparation. Three experiments were undertaken to evaluate the proposed technique. For all experiments, samples hydrolyzed as outlined in Chapter III, except the 50 mg bacterial samples which were hydrolyzed in 6 mL rather than 2 mL of 6 M HCl. A larger volume of acid was used to simplify subsequent dilutions and additions. Additional acid was not needed to complete bacterial hydrolysis.

In the first experiment, six levels of DAPA were analyzed to determine the lower limit of linear DAPA detection. Each level was analyzed in triplicate. Diaminopimelic acid levels analyzed are reported as though added to (blank) digesta. Levels of DAPA analyzed included .86, 1.72, 3.45, 6.89, 13.79 and 27.58 nmol per mg digesta. Internal standard (DL-α-amino-caprylic acid) was added at 21.6 nmol per mg digesta. In the second experiment standard additions of DAPA were made to hydrolyzed rumen digesta to determine if DAPA recovery would be affected when analyzed in a complex physiological sample. The digesta used was previously described in Chapter III. The analysis was completed in quadruplicate, generating four standard addition curves. The additions of DAPA included 0, 2.84, 5.67 and 8.53 nmol per mg of digesta. Internal standard was added at 21.6 nmol per mg of digesta.

The third experiment was designed to determine the repeatability of the technique by measuring the content of DAPA in six hydrolysates of rumen digesta and six hydrolysates of bacteria (both sample types were previously described in Chapter III). Preparation of hydrolysates prior to HPLC included the addition of 200 μl of water and 200 μl of 16 μmol per mL internal standard. Standard samples contained 6 mL of 6 M HCl, to which was added 200 μl of 32 μmol per mL DAPA and 200 μl of 16 μmol per mL internal standard. Hydrolysates and standards were then mixed and centrifuged for 15 min at 2500 *g*, and 10 μl of each were added and mixed with 10 μl of 6 M NaOH, 200 μl of saturated $K_2B_4O_7 \cdot 4H_2O$, and 750 μl of water. Final concentrations of digesta and bacteria immediately prior to OPA reagent addition were .230 and .077 mg per mL, respectively. Standards contained 10 nmol per mL DAPA, and all bacteria, digesta and standard samples contained 5 nmol per mL internal standard.

High-performance liquid chromatography. To complete the HPLC analysis of DAPA, 50 μl of OPA reagent solution were added and mixed with 75 μl of prepared hydrolysate or standard. Fifteen microlitres of derivatized sample were then injected and subjected to HPLC. O-phthaldialdehyde reagent addition and mixing, sample injection and HPLC were carried out using the equipment described in Chapter III. In addition, a pre-injection Supelco 50 x 4.6 mm I.D. guard column (Supelco, Bellefonte, PA, U.S.A.) containing pre-column silica gel (Whatman Biosystems, Maidstone, U.K.) was employed to protect C-18 packing in the post-injection columns. Peak heights and retention times were measured using a Shimadzu EZchrom Chromatography Data System (Shimadzu Scientific Instruments Inc., Columbia, MD, U.S.A.).

A binary gradient, changing from a polar to a non-polar solvent, was used for sample elution (Table VI.1). The polar solvent consisted of a water-methanol mixture (60:40, v/v) containing .1 M sodium acetate and 7.5 mM HTMA. The non-polar solvent consisted of a methanol-water mixture (95:5, v/v) containing 7.5 mM HTMA. The HTMA used should be recently manufactured. Aged HTMA stocks gave variable retention times and solubilized C-18 column packing. Both polar and non-polar solvents were adjusted to pH 6.4 with glacial acetic acid prior to their use. Under the conditions defined DD,LL-DAPA and DL-DAPA were found to elute at 30.3 and 31.8 min, respectively.

Statistical analysis. Data from experiment one, to determine the lower limits of linear DD,LL-DAPA and DL-DAPA detection, were analyzed as described in Chapter III. The quadratic components of both DD,LL-DAPA and DL-DAPA regression models were not significant ($P > .5$). To determine the analytical variation in experiment one, data were subsequently subjected to simple linear regression analyses (Steele and Torrie, 1980). To determine the consistency of DAPA recovery from rumen digesta, data from experiment two were subjected to simple linear regression analyses.

Repeatability of DAPA analyses was determined by calculating the SD and CV of DAPA concentrations found in experiment three.

C. RESULTS AND DISCUSSION

The ability of HTMA to preferentially slow the elution of the DAPA-OPA derivatives is clearly demonstrated when Figure III.1 and VI.1 are compared. To date, no other compounds of physiological origin have been detected near either DAPA peak, except for cystathionine. In case of cystathionine interference, the retention time of the parent molecule can likely be changed after oxidation with the method described by Moore (1963). DL-α-amino-caprylic acid has been found to be an excellent internal standard for all samples analyzed to date. If DL-α-amino-caprylic acid cannot be used, 1-amino-undecanoic acid and 1-amino-dodecanoic acid are possible internal standard alternates. Long chain diamino-alkanes (e.g., 1,10-diamino-decane) are not recommended as internal standards because their analyses were not found to be reproducible.

In the first experiment, neither the DD,LL-DAPA nor the DL-DAPA data sets exhibited a significant quadratic function over the concentrations tested ($P>.5$). A breakpoint between linear and non-linear detection could, therefore, not be established. Regressions of peak heights versus DD,LL-DAPA and DL-DAPA concentrations indicated little deviation from linear (DD,LL-DAPA, $r^2= 1.0000$; DL-DAPA, $r^2= .9999$). The smallest amount of DD,LL-DAPA or DL-DAPA actually injected and detected within the linear range was .90 ρmol, indicating the potential for analysis of less than 82 ng DD,LL-DAPA or DL-DAPA per mg of digesta.

In the second experiment, the four recovery curves of DAPA from rumen digesta yielded an average DD,LL-DAPA recovery of 99.3% (recovery range 98.8-100.1%; $r^2= .9998$-1.0000) and 98.4% for DL-DAPA (recovery range 96.9-100.0%; $r^2= .9993$-.9999). These results indicate that the effectiveness of the ion pairing agent is not impaired when physiological samples are analyzed. In the final experiment, to determine the repeatability of the method, mean DD,LL-DAPA and DL-DAPA quantities measured in digesta were .63 nmol per mg (SD ± .01, CV ± .85%) and 3.94 nmol per mg (SD ± .11, CV ± 2.9%), respectively. Levels of DD,LL-DAPA and DL-DAPA measured in the bacterial samples were 1.02 nmol per mg (SD ± .04, CV ± 3.9%) and 7.6 nmol per mg (SD ±

.14, CV ± 1.8%) respectively.

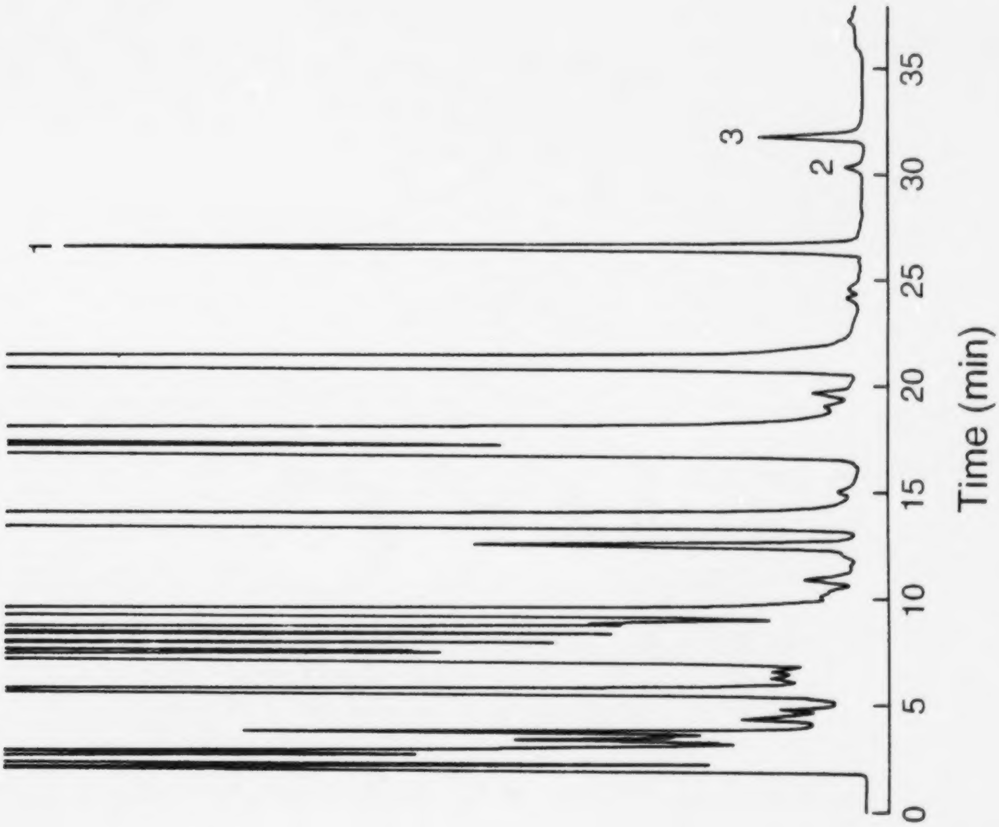
Experimental observations indicate that the method is sensitive, reproducible and easily implemented. It is less labour intensive than the method described in Chapter III and enables quantification of both DD,LL-DAPA and DL-DAPA. As indicated by Webster et al. (1989), measurement of only one of the two DAPA peaks is necessary for bacterial protein estimation, but being able to determine the DD,LL-DAPA to DL-DAPA ratio can yield important additional information. To obtain an accurate bacterial protein estimation, purified bacteria collected must be representative of the bacteria in the digesta. Different species of bacteria contain different DD,LL-DAPA and DL-DAPA concentrations. If the DD,LL-DAPA to DL-DAPA ratio is different for purified bacteria versus the digesta they were isolated from, the bacterial protein estimation may be incorrect. The ability to consistently measure both DD,LL-DAPA and DL-DAPA thus provides an additional advantage over previous methods for DAPA analysis.

TABLE VI.1. HPLC GRADIENT CONDITIONS[a]

Time (min)	Polar solvent (%)
0	50
1.0	40
32.5	19
33.0	0
36.0	0
37.0	50

[a] Flow rate: 1.1 mL/min; temp: 21 °C; run time: 38 min.

Figure VI.1. Chromatogram of the rumen bacteria hydrolysate. Peaks: 1 = DL-α-amino-caprylic acid; 2 = DAPA I: 3 = DAPA II.


1
2
3
0
5
10
15
20
25
30
35
Time (min)

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VII. GENERAL DISCUSSION AND CONCLUSIONS

The initial objective of these studies was to determine the bacterial contribution to crude protein (CP) in pig ileal digesta and feces using ^{35}S as a marker. A ^{35}S technique was used to try and avoid the large amount of variability reported when diaminopimelic acid (DAPA) was used as a bacterial marker (Drochner, 1984). Studies with sheep indicated ^{35}S to be the bacterial marker of choice when precise bacterial CP measurements needed to be made (Ling and Buttery, 1978). A novel double cannulation procedure was, therefore, developed (Chapter II) which enabled both the ^{35}S labelling of bacteria in the digestive tract and the collection of ileal digesta. A ^{35}S marker trial was then conducted in pigs fed either a purified (casein/cornstarch) or a conventional (barley/wheat/soybean meal) grower diet. Calculated bacterial CP expressed as a percentage of total CP in ileal digesta and feces exceeded 100% (in some instances over 500%). It was concluded that the ^{35}S marker technique, as used in this study, was not feasible for the determination of the bacterial contribution to CP in pig ileal digesta or feces. A second animal study was then planned (Chapter V) to determine the bacterial amino acid levels in pig ileal digesta using both a DAPA and a refined ^{35}S bacterial marker to enable comparison of both marker methods.

Only small bacterial pellets were collected in the first animal study (Chapter II); this indicated a need for a micro-method for DAPA analyses in the second animal study (Chapter V). Despite exhaustive manipulations to the method described by Jones and Gilligan (1983), for the high performance liquid chromatograph (HPLC) analysis of amino acids, it was not possible to consistently separate the DAPA peaks (DD,LL-DAPA and DL-DAPA) from co-eluting peaks. A review of potential cleanup procedures indicated that DAPA had an exploitable affinity for cellulose. A method, reported in Chapter III, was developed for the cellulose cleanup of hydrolysates of physiological samples which was coupled with a modified Jones and Gilligan (1983) method to enable the sensitive analysis of DL-DAPA in milligram quantities of bacteia and digesta.

In the first animal study (Chapter II) samples were analyzed in oversized vessels (250 mL round bottom flasks) due to the lack of appropriate drying equipment for use with test tubes. Furthermore, sample preparation was slow due to the use of roto-evaporation to dry samples. A test tube dryer was needed that was rapid, inexpensive and resistant to corrosive $Br_{2(g)}$. Such a dryer was developed and is described in Chapter IV. Under conditions defined in Chapter IV, the dryer was found to be capable of evaporating water from a 16 x 150 mm test tube at the rate of 42.6 mL per h.

The technical developments reported in Chapters III and IV were implemented in the second animal study described in Chapter V. Methods used in the original ^{35}S study (Chapter II) were modified and incorporated into the second animal study to overcome postulated ^{35}S labelling errors. Changes to the method were, however, unable to rectify overestimation of the bacterial contribution to amino acids in ileal digesta when the ^{35}S marker was used. Based on ^{35}S incorperation, bacterial amino acids accounted for 191% of total amino acids in ileal digesta. Based on DAPA, however, the content was estimated to be 29.4%. Possible explanations and remedies for the failure of the modified ^{35}S technique were again proposed. The most promising remedy included the isolation of a pure ^{35}S marker fraction from bacteria and digesta by reacting and extracting the methane thiol group from sample methionine using the CNBr technique described by Paul (1977).

Further calculations based on the DAPA marker technique revealled some interesting details concerning the bacterial amino acid content in ileal digesta. Bacterial amino acids were found to significantly affect ($P < .05$) the content of five of the 16 amino acids (isoleucine, aspartic acid, glycine, cysteine and tyrosine) in digesta. In addition, when bacterial amino acid levels in ileal digesta changed within pig over time, the undigested endogenous and dietary residual amino acids levels were found to change in the same direction. These results indicate that bacteria can affect the supply of amino acids to the pig, not only by retaining amino acids, but also by modifying the amino acid profile in digesta and influencing the digesta content of undigested dietary and endogenous residuals. Bacteria in ileal digesta, however, only contained approximately 5% of the amino acids consumed. Their effect on the total supply of dietary amino acids may thus be small, but these minor losses would likely be economically significant on a farm or industry scale.

As was indicated in Chapter V, some difficulties were encountered with the analysis of DL-DAPA in the dual marker study. The HPLC solvent gradient had to be changed and it was necessary to use external standards due to the presence of compounds that co-eluted with the internal standard and DL-DAPA. The development of an alternative method for DAPA analysis was thus undertaken to provide simpler more routine analyses of DAPA for future animal experiments. Instead of removing co-elutants by using a cleanup procedure, it was decided to investigate the use of an ion-pairing agent in the solvent system to selectively modify the chromatographic characteristics of the DAPA/o-phthadialdehyde (OPA) derivatives. A method was developed, as described in Chapter VI, using the cationic detergent hexadecyl-trimethyl-ammonium bromide to ion-pair with the DAPA-OPA derivatives to enable their selective retention on a reverse phase HPLC column. This method was shown to have the potential for the detection of less than 82 ng of either DD,LL-DAPA or DL-DAPA per mg of sample. The fact that both DAPA peaks can be consistently measured imparts an added advantage to the method. The peak ratio can be used in future exeriments as an internal control to determine if the bacteria collected from ileal digesta are representative of bacteria in the whole digesta.

In conclusion, it must be emphasized that the DAPA marker results from the second animal trial must be taken cautiously due to their disagreement with the ^{35}S marker results. Further research is clearly needed in this area and it is hoped that these studies have helped form the foundation for such research. An effort to determine the content of endogenous, bacterial and dietary amino acids in pig ileal digesta using both the ^{15}N-leucine isotope dilution technique (de Lange, 1992) and the DAPA marker technique is currently in progress.

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